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(71) Applicant: GEORGETOWN UNIVERSITY [US/US]; Office of Financial Affairs, Ryan Administration Building, 37th & O Streets, N.W., Washington, DC 20057 (US).

(72) Inventors: LIPPMAN, Marc ; 8004 Herb Farm Drive, Bethesda, MD 20817 (US). LUPU, Ruth ; 1737 Yale Place, Rockville, MD 20850 (US).

(74) Agents: BEAUMONT, William, E. et al.; Oblon, Spivak, McClelland, Maier & Neustadt, Fourth Floor, 1755 South Jefferson Davis Highway, Arlington, VA 22202 (US).

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(54) Title: GROWTH FACTOR WHICH INHIBITS THE GROWTH OF CELLS OVEREXPRESSING THE HUMAN ONCOGENE erbB-2

(57) Abstract

A method for inhibiting the growth of adenocarcinoma cells in a human, which cells overexpress the oncogene erbB-2, which entails administering to said human an amount of a 30 Kd glycoprotein effective to inhibit the growth of said cells.

-1-
Description

Growth Factor Which Inhibits the Growth
of Cells Overexpressing the Human Oncogene erbB-2

Technical Field

- 5 The present invention relates to a growth factor which interacts with the human oncogene erbB-2, and which inhibits the growth of cells overexpressing this oncogene.

Background Art

- 10 Carcinogenesis is believed to be a multi-step process of alteration of genes which are involved in the growth control of cells. A variety of proto-oncogenes and oncogenes have been implicated in the activation of tumor cells as regulating factors. For example, oncogenic protein kinases are believed to induce cellular transformation
15 through either inappropriate or excessive protein phosphorylation, resulting in the uncontrolled growth of malignant neoplasms. See Histopathology, Wrba, F. et al, 15, 71-76 (1989).

- 20 One group of proto-oncogenes encodes cellular growth factors or their receptors. The c-erbB-1 gene encodes the epidermal growth factor or their receptors. The c-sis gene encodes the B-chain of the platelet-derived growth factor. The c-fms gene encodes a related or identical molecule for the receptor of the granulocyte-macrophage colony
25 stimulating factor. A fourth member of this group of proto-oncogenes, called neu was identified in ethylnitrosourea-induced rat neuroblastomas. The human counterpart of neu, called HER-2/neu or c-erbB-2, has been

sequenced and mapped to the chromosomal locus 17q21. See Cancer Research, Schneider, P.M. et al, 49, 4968-4971 (September 15, 1989).

5 The HER-2/NEU OR c-erbB-2 oncogene belongs to the erbB-like oncogene group, and is related to, but distinct from the epidermal growth factor receptor (EGFR). The oncogene has been demonstrated to be implicated in a number of human adenocarcinomas leading to elevated levels of expression of the p185 protein product. For example, the
10 oncogene has been found to be amplified in breast, ovarian, gastric and even lung adenocarcinomas.

Furthermore, the amplification of the c-erbB-2 oncogene has been found in many cases to be a significant, if not the most significant, predictor of both overall survival time
15 and time to relapse in patients suffering from such forms of cancer.

Carcinoma of the breast and ovary account for approximately one-third of all cancers occurring in women and together are responsible for approximately one-fourth of
20 cancer related deaths in females. Significantly, the c-erbB-2 oncogene has been found to be amplified in 25 to 30% of human primary breast cancers. See Science, Slamon, D. et al, 244, 707-712 (May 12, 1989).

25 The c-erbB-2 oncogene is known to express a 185Kd transmembrane glycoprotein (p185^{erbB-2}). The expressed protein has been suggested to be a growth factor receptor due to its homology with EGFR. However, known EGFR ligands, such as EGF or TGF α , do not bind to p185^{erbB-2}. At present,

no ligand is known which binds to this protein.

Thus, a need continues to exist for a ligand for (p185^{erbB-2}). Such a ligand might be used to counteract the effects of c-erbB-2 oncogene over expression in facilitating carcinogenesis.

Disclosure of the Invention

Accordingly, it is an object of the present invention to provide a growth factor which interacts directly with the erbB-2 oncogene.

10 It is also an object of the present invention to provide a method for the isolation and purification of the abovedescribed growth factor.

It is also an object of the present invention to provide a method for inhibiting the growth of cells which overexpress the human oncogene erbB-2.

15 Accordingly, the above objects and others are provided by a 30kDa TGF α -like glycoprotein.

Brief Description of the Drawings

20 Figure 1 illustrates the isolation of the present 30 Kd growth factor. Portion A illustrates the use of low affinity heparin chromatography, while portion B illustrates the use of reversed-phase chromatography.

Figure 2 illustrates the detection of phosphorylated

proteins in SK-Br-3 cells.

Figure 3 illustrates the detection of phosphorylated proteins in MDA-453 cells.

Figure 4 illustrates the phosphorylation of
5 p185^{erbB-2} protein in intact CHO/DHFR and CHO/erbB-2 cells.

Figure 5 illustrates a p185^{erbB-2} receptor competition assay in SK-Br-3 cells.

Figure 6 illustrates the inhibition of p185^{erbB-2} crosslinking with 4D5 antibody by gp30.

10 Best Mode for Carrying Out the Invention

The human c-erbB-2 oncogene encodes a 185 Kd transmembrane glycoprotein having protein kinase activity. This glycoprotein, known as p185^{erbB-2}, shows extensive structural similarity with the p170 epidermal growth factor
15 receptor (EGFR) and is therefore thought to be growth factor receptor. However, neither EGF nor TGF α , the normal ligands for the EGFR, interact directly with p185^{erbB-2}. In fact, no ligand for this glycoprotein has been described. It would be extremely desirable to find a ligand for this 185 Kd
20 glycoprotein inasmuch as erbB-2 oncogene is amplified in many adenocarcinomas and is over expressed in nearly 30% of human breast cancer patients. Additionally, it is known that p185^{erbB-2} is necessary for the maintenance of the malignant phenotype of cells transformed by the oncogene.

25 In accordance with the present invention, it has been

surprisingly discovered that a 30 Kd growth factor which is secreted from the estrogen receptor negative cell line MDA-231 is effective as a ligand for p185erbB-2 glycoprotein. The 30 Kd glycoprotein of the present invention also exhibits TGF α -like activity. For example, the present 30 Kd glycoprotein binds to EGFR, is capable of phosphorylating EGFR as well as inducing NRK colony formation. This is quite surprising inasmuch as the present 30 Kd growth factor is distinct from the normal precursor for TGF α or mature TGF α as shown by peptide mapping of the translated proteins.

Generally, the 30 Kd glycoprotein was immunoprecipitated by an anti-TGF α polyclonal antibody and exhibited TGF α -like biological activity as assayed by EGF radioreceptor assay and NRK and A1N4T cell colony formation assays. The 30 Kd growth factor also stimulated autophosphorylation of the EGF receptor more efficiently than mature 6 Kd TGF α .

The 30 Kd glycoprotein was observed, unlike EGF and TGF α , to bind to heparin-sepharose, and was purified to apparent homogeneity by heparin affinity chromatography and subsequent reversed phase chromatography.

Tunicamycin treatment in vivo or N-glyconase deglycosylation in vitro revealed a precursor of 22 Kd in contrast to the 18 Kd precursor for mature TGF α . Furthermore, in vitro translation of total mRNA from MDA-MB-231 cells confirmed these observations. Biochemical characterization of the 30Kd TGF α -like protein was obtained by V8-protease digestion of the de-glycosylated polypeptides

and translated products. Peptide mapping of the V8-digested, immunoprecipitated material suggests an amino acid sequence distinct from TGF α . Hence, the 30Kd polypeptide, while related to the EGF/TGF α family, is encoded by a different gene and is not a post-translation modification of mature TGF α .

Having obtained the present 30 Kd glycoprotein, in accordance with another aspect of the present invention, the same is used to inhibit the growth of cells which overexpress the c-erbB-2 oncogene.

In accordance with the present invention, the present 30 Kd glycoprotein may be used, by itself, or in conjunction with other medicinal substances to inhibit the growth of any cells which overexpress the c-erbB-2 oncogene.

Generally, the present 30 Kd glycoprotein may be used advantageously to inhibit the growth of adenocarcinoma cells, preferably those of breast, ovarian, gastric and lung tissue which overexpress the erbB-2 oncogene and EGFR.

In order to further exemplify the present invention, reference will now be made to certain examples which are provided solely for purposes of illustration and are not intended to be limitative.

MATERIALS AND METHODS FOR OBTAINING THE 30 Kd GLYCOPROTEIN

Cell Lines

Cells from the following sources were used: MDA-MB-231

and NRK clone 49F fibroblasts were obtained from the American type Culture Collection (Rockville, MD). Hs578T cells, A431 cells, and H8 cells, a TGF α -transfected MCF-7 breast cancer cell line, were available upon request from a variety of sources. Carcinogen-immortalized normal mammary epithelial cell subline 184A1N4 and its SV40-transfected derivative 184A1N4T, were also available on request. Rat-FeSV transfected cells were also provided upon request. All cell lines were propagated in improved modified Eagle's medium (IMEM, Gibco, Grand Island NY) supplemented with 10% fetal bovine serum (FBS, Gibco).

Conditioned Media Preparation, Collection and Concentration

Conditioned media collections were carried using a well-known procedure. The media were concentrated 100-fold in an Amicon ultra-filtration cell (YM5 membrane) (Amicon, Danvers, MA). Once clarified and concentrated, the media were stored at -20°C while consecutive collections were made during the following days. The concentrated media were dialyzed using Spectraphore 3 tubing (Spectral Medical Industries, Los Angeles, CA) against 100 volumes of 0.1 M acetic acid over a two day period at 4°C. The material that precipitated during dialysis was removed by centrifugation at 4000 rpm for 30 min. at 4°C; protease inhibitors were added. The clarified sample was then lyophilized.

Metabolic Labelling and Immunoprecipitation

Cells were grown to 80% confluence in IMEM. Cell monolayers were washed three times with PBS and incubated

for two hours in serum-free IMEM which lacked methionine and cysteine and was supplemented with glutamine (2.9 g/l) (Biofluids, Rockville, MD). This medium was then removed and replaced with serum-free IMEM without methionine and cysteine containing 2.5 mCi/ml [³⁵S] cysteine and methionine (Amersham, Arlington Heights, IL, 1175 Ci/mmol). A total of 2.5 ml of this medium was used for a 5 cm dish. The medium was harvested from the culture after 16 hrs at 37°C and clarified by centrifugation. Cells were washed once with PBS, harvested by scraping, and lysed in 1 ml of RIPA buffer (300 mM NaCl, 100 mM Tris-HCl, containing 2% Triton x100, 2% Nadeoxycholate, 0.2% SDS, 0.4% BSA and 2 mM PMSF). Following an incubation of 30 minutes on ice, the lysate was clarified by centrifugation (30 minutes at 4000 rpm) and used immediately or was stored at -70°C. [³⁵S]-labelled proteins released into the conditioned media by the different cell lines were immunoprecipitated with 10 g (specific or non specific) antibody partially purified by 45% ammonium sulfate precipitation. After solubilization the immunoprecipitates were analyzed by 15% SDS-PAGE and subsequent fluorography. Prestained molecular weight markers (Biorad, Richmond, CA) were run in parallel lanes.

Tunicamycin Treatment

Tunicamycin (Sigma, St. Louis, MO) was dissolved in 50 mM sodium carbonate (pH 10.0) and filter-sterilized with a 0.22 m filter. Confluent monolayers of MDA-MB-231, MCF-7 and Hs578T cells were grown in IMEM in the presence of 20 g/ml tunicamycin (unless otherwise specified) for 4 hours prior to metabolic labelling. Metabolic labelling was then performed as described above with continued tunicamycin

treatment.

Elastase Treatment

The samples containing TGF α -like activity were incubated with 20 g porcine pancreatic elastase (Sigma) dissolved in 50 mM glycylglycine, pH 7.9, for 1 hour at 22°C. The samples were then subjected to immunoprecipitation and SDS-PAGE analysis.

Polyclonal and Monoclonal Antibodies

Polyclonal Antibodies; Antiserum against human TGF α was obtained by immunization of a rabbit on day 0 with 400 g of recombinant TGF α synthesized in *E. coli*, provided by Genentech Corp. The immunogen was first conjugated to keyhole limpet hemocyanin (KLH) and was emulsified in complete Freund's adjuvant and was injected intradermally at multiple sites. Additional injections were given as follows: day 60, 175 g TGF α and days 90, 150, 180, and 210, 100 g TGF α . The booster injections were given subcutaneously at multiple sites in incomplete Freund's adjuvant. The rabbit serum was assayed for antibody titer by ELISA at 10 to 14 days following each injection. The antiserum collected at day 180, designated R399, was used for immunoprecipitation and radioimmunoassay.

Monoclonal Antibodies; A monoclonal antibody against recombinant TGF α was kindly provided by Genetech Corp.

Measurement of Anti TGF α Antibody (R.399) Levels by ELISA
Micro-Elisa plates (Dynatech-Immunolon II, Dynatech

Laboratories, Inc. Chantilly VA.) were coated for 16 hours at 4°C with 500 ng/ml of recombinant TGF α in 50 mM sodium - carbonate buffer (pH 9.6). The samples to be assayed (antibody) were serially diluted 1:1,000-1:64,000 with 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4), 2 mM EDTA, 5 mg/ml bovine serum albumin, 0.05% Tween 20 (TBS-BSA-Tween) and were incubated in the wells for 2 hours at 37°C. The plates were washed five times with PBS-Tween and then incubated for 1 hr at 37°C with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin in TBS-BSA-Tween. The plates were then washed five times with PBS-Tween and incubated for 4 hrs at 22°C with 100 μ l per well of 0.1 mg/ml o-phenylenediamine, 0.012% H₂O₂ in 0.1 M Phosphate-citrate buffer (pH 5.0). The reaction was stopped by the addition of 50 μ l/well of 2.5 N H₂SO₄ and the absorbance was measured at 492 nm using a UR 700 Microplate Reader (Dynatech Lab., Inc. Chantilly, VA).

Radioimmunoassay (RIA)

TGF α RIA; The presence of peptides immunologically related to TGF α was determined using a RIA kit with a polyclonal anti-rat TGF α and rat [²⁵I]TGF α (Biotope, Inc., Seattle, WA). This antibody does not cross-react with human EGF. Aliquots of conditioned media were reduced with 40 mM dithiothreitol and denatured by immersion for 1 minute in a boiling water bath. Assays were done in duplicate according to the manufacturer's protocol and each collection of conditioned media was assayed at least twice.

Solid Phase RIA; 96 well microtiter plates were coated with anti-TGF α antibody (R399 or monoclonal antibody) for 2

hours at 37°C. The wells were then filled with 100 μ l of the column fraction to be assayed for TGF α activity. A standard curve was constructed using 0.075 to 15 ng unlabelled TGF α . After the 2 hours incubation 5×10^4 cpm of [125 I] TGF α or 5 2×10^5 cpm of metabolically labelled antigen was added per well. The plates were incubated further for 16 hours at 4°C. The wells were then washed and counted using a gamma counter (Model B5002, Packard Instruments Co., Sterling, VA). The EGF RIA was performed with an anti-EGF antibody 10 (Oncogene Science clone 144-8, Manhasset, NY). A standard curve was constructed using human EGF (HEGF, receptor grade, Collaborative Research, Waltham, MA).

N-Glycanase Digestion

The purified 30 kDa TGF α -like protein was subjected to 15 digestion with N-glycanase. Samples equivalent to 100 ng were incubated with 50 μ l of 0.2 M sodium phosphate (pH 8.6), 1.25% NP40 and 2-6 μ g N-Glycanase (Genzyme Corp., Boston, MA) were subsequently added to each sample and incubated at 37°C for 16 hours. 50 μ l of 3-fold concentrated loading buffer 20 was added before electrophoretic analysis, performed as outlined above. The gel was silver stained.

EGF Radioreceptor Assay

A431 membranes were prepared according to the method of Kimball and Warner. A431 cells were disrupted under 25 nitrogen and the nuclei and organelles pelleted by low speed centrifugation. The membranes were then pelleted by centrifugation at 35,000 rpm for 1 hour and resuspended in - 20 mM HEPES buffer, pH 7.4. Membranes (2.5 g/ml) were

plated into 96 well plates and allowed to dry overnight at 37°C before use. Standard binding competition studies were performed using [125 I]EGF (ICN, Costa Mesa, California, specific activity- 100 Ci/g, about 50,000 CPM/well). A standard curve was constructed with 0.075-10ng of unlabelled hEGF (receptor grade, Collaborative Research). The different fractions to be analyzed were lyophilized and reconstituted in PBS (0.5 ml/500 ml conditioned media). After incubation of the labelled EGF and 10¹ of the samples for 2 hours at 37°C in binding buffer (IMEM containing 50 mM HEPES and 0.1% BSA pH 7.7), the wells were washed, cut from the plate and counted. EGF-competing activity, was computed using a Hewlett Packard RIA Program.

Anchorage-independent Growth Assay

Soft agar cloning assays were carried out using a 1 ml bottom layer of IMEM containing 0.6% Bacto-agar (Difco, Detroit, MI), 10% FBS, and 2 mM glutamine in 35mm tissue dishes (Costar, Cambridge, MA). A 0.8 ml top layer of IMEM containing the test samples, 0.36% agar, 10% FBS, and 3 x 10⁴ NRK cells was added after solidification of the bottom layer. Each sample was plated in triplicate. All samples were sterilized by filtration using a 0.22 μ Millex CU millipore filter before plating. Plates were incubated in a humidified, 5% CO₂ atmosphere at 37°C and were counted after 12 days incubation with a Bausch and Lomb Stem Cell Colony Counter (Artex Systems Corp, Farmingdale, NY).

Anchorage-dependent Growth Assay

Cells were grown in IMEM containing 5% FCS. Upon

confluence cells were detached using trypsin-versene (Biofluids, Rockville MD) and passed at 1:20 to 1:50 dilutions. Cells were seeded in 12-well plates at 4,000-10,000 cells/well, depending on the cell type

5 (MDA-MB-231-8,000 cells/well in serum free IMEM). After 24 hours the media was changed and the cells were treated with EGF, TGF α or TGF α -like protein were harvested at 1, 2 and 4 days using trypsin-versene. The cells were counted using a coulter counter.

10 Heparin Affinity Chromatography

Media conditioned by MDA-MB-231 cells were clarified by centrifugation for 20 minutes at 2,000 rpm at 4°C. The supernatant was collected and stored at -70°C. After allowing the heparin-sepharose (Pharmacia, Piscataway, NJ)

15 to expand in PBS, 2 ml of gel was loaded on an Econo column (Biorad, Richmond, CA) and washed with about 100 bead volumes of PBS. Conditioned media were run through the beads by gravity (flow rate 20 to 50 ml/hr). The gel was then washed with 5 volumes of PBS and eluted stepwise with

20 an increasing gradient of NaCl in 10 mM Tris-HCl, pH 7.0 (elution buffer). Gradient steps of 0.4 M, 1.1 M, 2.0 M and 3.0 M NaCl were used in the elution buffer until the 280nm absorption during each step returned to baseline (usually 3 to 5 column bed volumes). The eluate was desalted on G-25

25 columns (Pharmacia, Piscataway, NJ) and filter-sterilized before use in the different bioassays. Pooled fractions containing active materials were also desalted on PD10 columns (Pharmacia, Piscataway, NJ) before running through HPLC and FPLC.

Molecular Filtration Chromatography

Lyophilized conditioned medium was dissolved in 1 M acetic acid to a final concentration of about 25 mg/ml total protein. Insoluble material was removed by centrifugation at 10,000 rpm for 15 minutes. The sample was then loaded onto a Sephadex G-100 column (XK 16, Pharmacia, Piscataway, NJ), was equilibrated and was subjected to elution with 1 M acetic acid at 4°C with an upward flow of 30 ml/hr. 100 ng of protein was processed from 4 ml of 100-fold concentrated medium. Fractions containing 3 ml of eluate were lyophilized and resuspended in 300 μ l PBS for assay and served as a source for further purification.

Reversed-phase High Pressure Liquid Chromatography (HPLC)

Steep Acetonitrile Gradient; Step acetonitrile gradient and all other HPLC steps were carried out at room temperature after equilibration of the C3-Reversed phase column with 0.05% TFA (Trifluoroacetic acid) in water (HPLC-grade). The samples were loaded and fractions eluted with a linear gradient (0-45% acetonitrile in 0.05% TFA) at a flow rate of 1 ml/min over a 30 minute period. Absorbance was monitored at 280nm. One ml fractions were collected and lyophilized before analysis for EGF receptor-competing activity.

Shallow Acetonitrile Gradient; The pool of active fractions from the previous HPLC step was rechromatographed over the same column. Elution was performed with a 0-18% acetonitrile gradient in 0.05% TFA over a 5 minute period followed by a linear 18-45% acetonitrile gradient in 0.05%

TFA over a 30 minute period. The flow rate was 1.0 ml/min and 1 ml fractions were collected. Human TGF α -like factor was eluted at a 30-32% acetonitrile concentration as a single peak detectable by RRA.

5 Electrophoretic Elution of Radiolabelled Protein from Gels

After fluorography of an SDS-PAGE, bands of interest were excised and the protein eluted by electrophoresis into a dialysis tubing over 16 hrs at 120 volts. The contents of the dialysis bag were cooled at 4 C and then precipitated by
10 the addition of trichloroacetic acid to a final concentration of 20%. The precipitates were pelleted by centrifugation, washed twice with ethyl ether, and resuspended in loading buffer.

Digestion Procedure for Purified Eluted Proteins

15 Electroeluted proteins were dissolved at approximately 0.5 mg/ml in loading buffer which contained 0.125 M Tris-HCl (pH 6.8), 0.5% SDS, 10% Glycerol and 0.001% Bromophenol Blue. The samples were then heated at 100°C for 5 minutes. Proteolytic digestion were carried out at 37°C for 30
20 minutes by the addition of Staphylococcus aureus Protease V8 (Sigma, St. Louis, MO) to a final concentration of 25 g/ml according to methods. P-mercaptoethanol and SDS were subsequently added to final concentrations of 20% and 2%, respectively. Proteolysis was stopped by boiling for 2 min.
25 The samples were then injected on a C18 Reversed Phase HPLC column.

Phosphorylation of the EGF Receptor

Subconfluent A431 cells were cultured in IMEM for 10-12 hours. The cells were treated with 10-30 nM TGF α , EGF or TGF α -like growth factor for 30 minutes at 37°C. Cells were lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM EDTA, 2 mM PMSF, 42 mM leupeptin and immunoprecipitated as described above using monoclonal antibody 225 directed against the EGF receptor (Oncogene Science, Manhasset, NY). The immunoprecipitates were washed three times with RIPA buffer and resuspended in 40 μ l TNE (0.01M Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA). Five Ci of [γ -³²P]ATP was added to the immunoprecipitates and the total ATP concentration was adjusted to 15 mM (final) in a volume of 60 μ l. The reaction mixture was incubated for 5 minutes on ice before addition of 20 μ l of 3x sample buffer. The samples were boiled for 5 minutes and analyzed by denaturing 7.5% SDS-PAGE.

RNA Extraction

Total cellular RNA was extracted from cells by homogenizing in guanidine isothiocyanate followed by centrifugation over a cesium chloride cushion. Poly (A)⁺ mRNA was eluted in 10 mM Tris after passing total cellular RNA over an oligo (dT) cellulose column (Pharmacia, Piscataway, NJ) equilibrated with 10 mM Tris-0.5 M NaCl pH 8.0. After precipitation in ethanol (66% vol/vol) and 0.1 M acetic acid, both total and poly(A)⁺ selected RNA were resuspended in 10 mM Tris-1 mM EDTA buffer and separated on 1% agarose, 6% formaldehyde gels. Electrophoresis was carried out at 20 volts over 14-16 hours in: 5 mM NaAc 1 mM EDTA, 20 mM 3-[N-morpholino] propane sulfonic acid pH 7.0 (MOPS-Sigma). The gels were stained with ethidium bromide 2.0g/ml to allow inspection of the quality and quantity of

RNA (). In vitro translation assays were performed using Weat Germ kit according to the manufacturer's instructions (Promega).

5 Identification of a TGF α -like Polypeptide in MDA MB 231
Human Breast Cancer Cells

To determine whether the 30 Kd TGF α -like protein was recognized by antibodies developed against mature 6 kDa TGF α , MDA-MB-231 cells were metabolically labelled with [35S] methionine and [35S] cysteine. Metabolically labelled
10 conditioned media from MDA-MB-231, TGF α -transfected MCF-7 (H8), and HS578T cells were tested by solid phase RIA for immunoreactivity with a polyclonal antibody (R399) and a monoclonal antibody raised against recombinant 6 kDa TGF α . Metabolically labelled TGF α -like material from MDGA-mb-231
15 cells reacted only with the polyclonal antibody. In contrast, the two antibodies cross-reacted with metabolically labelled material derived from H8 cells and no immune-reaction was noted with preimmune serum (normal rabbit serum NRS) or metabolically labelled conditioned
20 media from Hs578t breast carcinosarcoma cells (Fig. 1), which do not produce TGF α mRNA. Thus only the monoclonal antibody is able to distinguish between the different species of TGF α . Specificity of the assay was demonstrated using a competition RIA with unlabelled recombinant TGF α .

25 Labelled material from MDG-MG-231, H8, and Rat-FeSrv cells was immunoprecipitated with the anti-TGF α polyclonal antibody. Detection of an immunoreactive species of approximately 30 kDa size verified the secretion of a high molecular weight TGF α -like polypeptide in MDA-MB-231 cells.

H8 cells, which overexpress classical TGF α , yielded a 6 kDa product. The expected 18 kDa precursor of the classical 6 kDa TGF α was precipitated from Rat-FeSrv, which are known to secrete the "normal" precursor. The intensity of the bands
5 diminished when the immunoprecipitation was performed in the presence of excess unlabelled TGF α . No specific bands were immunoprecipitated by preimmune rabbit serum.

The Larger TGF α -like Species Is Glycosylated

The apparent heterogeneity in size of the larger TGF α species and the potential for N-linked glycosylation of the
10 TGF α precursor at Asn 25 led to the consideration of whether the high molecular weight TGF α -like polypeptide secreted from MDA-MB-231 cells was a glycosylated form of TGF α . When MDA-MB-231 cells were incubated with tunicamycin, an
15 inhibitor of co-translational N-linked glycosylation, and the media was immunoprecipitated with the anti-TGF α polyclonal antibody a species of 22 kDa substituted that previously observed of 30 kDa. Additional cleavage of the 22 kDa polypeptide with elastase yielded an apparent 11 kDa
20 product different from the mature 6 kDa TGF α that was observed in Rat-FeSrv labelled media. The 11 kDa product had a higher immunoreactivity with the R399 antibody than the 30 kDa and the 22 kDa polypeptides. Shorter exposure of the gel showed clearly a precipitated band near the 11 kDa
25 molecular weight. Tunicamycin treatment did not significantly affect the levels of secreted TGF α activity as determined by both RIA and EGF receptor binding assays.

When the purified 30 kDa polypeptide was treated with Nglyconase a 22 kDa product was detected by silver staining.

The absence of cleavage of the purified 30 kDa polypeptide after O-glyconase treatment suggests that no O-glycosylation occurs in this system.

Purification of the TGF α -like Polypeptide

5 TGF α -like, material was isolated from serum-free conditioned media of MDA-MB-231 cells. Levels of TGF α -like polypeptide were quantified by three independent assays: capacity to induce anchorage-independent growth of NRK
10 fibroblasts in soft agar, ability to compete with (125 I)EGF for EGF receptor binding on A431 human carcinoma cell membranes and cross-reactivity with polyclonal antibodies raised against mature TGF α . EGF receptor binding activity and TGF α immunoreactivity were detected using a RIA kit provided by Biotops. To determine the approximate molecular
15 weight of the MDA-MB-231 derived TGF α -like polypeptide, 5 ml of 100-fold concentrated, dialyzed conditioned medium was chromatographed by gel filtration using Sephadex G-100. Elution was performed with 1.0 M acetic acid and fractions were characterized for protein content. TGF α -like
20 activities were eluted from the column in a single broad peak. Maximal activity was observed at an apparent molecular weight of 30 kDa and was separated from the bulk of contaminating proteins present in the bed volume. All the fractions demonstrating TGF α immunoreactivity also
25 contained EGF receptor binding activity. The relative amounts of receptor binding activity and immunoreactivity present in these fractions, however appeared to differ. Further analysis of the TGF α -like polypeptide from MDA-MB-231 cells was carried out using heparin-sepharose
30 affinity chromatography. Heparin-sepharose affinity

chromatography was performed on unconcentrated conditioned media from MDA-MB-231 cells. In all experiments, less than 20% of the TGF α activity loaded onto the column was recovered in the unabsorbed fractions. A sharp peak of EGF receptor binding activity was eluted by heparin-sepharose chromatography at a concentration of 0.4-0.5 M NaCl. This activity represented one major 30 kDa molecular weight protein, which retained 70%-80% of the load activity.

The TGF α -like polypeptide was further purified by reversed phase chromatography (HPLC) in two steps. A pool of fractions containing EGF receptor-competing activity from heparin-sepharose chromatography was reconstituted in 0.05% TFA in water and then chromatographed on a Bondapak C₃ column. A steep acetonitrile gradient (0-100%) was used in this step. TGF α -like polypeptide elutes as a sharp peak in 30% acetonitrile and is separated from the bulk of the contaminating proteins. The capacity of the individual fractions to compete for EGF receptor binding and to stimulate the growth of NRK cells in soft agar was determined. A pool of the active fractions (indicated with an horizontal bar) was rechromatographed on the same column. Fractions were eluted with a 0-20% acetonitrile gradient in 0.05% TFA for 5 minutes followed by a linear 20-40% acetonitrile gradient. The TGF α -like polypeptide activity was eluted at 25-30% acetonitrile and effectively separated from other contaminant proteins.

In order to achieve a complete separation of TGF α -like polypeptide from those impurities detected by silver staining (data not shown) we used size exclusion chromatography under acidic conditions. The active

fractions for EGF receptor-competing activity were pooled and analyzed by SDS-PAGE. One single polypeptide band was observed after silver staining.

- 5 A summary of the steps leading to the isolation and purification of TGF α -like polypeptide is presented in Table 1. A 27% recovery of activity and approximate 5400 fold purification was achieved.

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Table 1: Purification of TGF α -Like Activity from Conditioned Medium from MDA-MB-231 Cells

Purification step	Protein Recovered ^a (mg)	EGF competing activity (units/mg TGF α)	Relative Specific Activity (units/mg protein)	Degree of Purification (fold)	Recovery (% Activity)
Conditioned Medium	96	450	4.6	1	100
Acid-Soluble Supernatant	82	419	5.1	1	93
Gel Filtration	2.95	209	70.8	15.3	46
1- Heparin-Sepharose	1.54	230	149	32.3	51
2-Reverse Phase ^c	0.03	173	5766	1253	39
3-Reverse Phased	0.005	124	24800	5400	27

a. Total protein was determined using BSA as a standard. The quantitation of step 6 was based on extrapolation from standard values. The absolute specific activity of a companion aliquot was found to be 1 million Units/mg.

b. One unit of EGF competing activity is defined as the amount of protein that inhibits the binding of [¹²⁵I]EGF to the receptor by 50%.

c. Steep acetonitrile gradient.

d. Shallow acetonitrile gradient.

1-2-3: Subsequent purification steps

* Each value represents the mean of 4-6 experiments and they were reproducible with in 10%.

Biological Characterization of the TGF α -like Material

The EGF receptor binding activity of the 30 kDa TGF α -like protein was compared with that of EGF in a radioreceptor assay. Both growth factors competed with [125I]EGF for receptor sites on A431 membranes. The specific EGF-competing activity of the purified TGF α -like polypeptide was found to be $1-1.5 \times 10^6$ units/mg; 1.1ng of TGF α -like polypeptide was required to inhibit EGF binding by 50%. TGF α -like polypeptide was as effective as EGF in EGF receptor binding. Furthermore, the purified 30 kDa TGF α -like polypeptide stimulated the growth of serum NRK fibroblasts and induced colony formation of these cells in soft agar. The bioactivity of the purified TGF α -like polypeptide was also tested by anchorage-dependent growth assays of the carcinogen-immortalized human mammary epithelial cells 184A1N4 and anchorage-independent growth assays of 184A1N4-derived cells partially transformed by SV40 T antigen, 184A1N4T. Dose response curves of TGF α -like polypeptide on these cells were similar to those observed with EGF and TGFU. The biological activity of the purified 30 kDa TGF α -like factor was further assessed by examining its ability to induce autophosphorylation of the EGF receptor. A431 cells, which overexpress the EGF receptor, were incubated with various concentrations of EGF, TGFU or TGF α -like growth factors. Each of the three peptides similarly stimulated phosphorylation of the EGF receptor.

Peptide Mapping

In order to determine the degree of homology between the novel 30 kDa TGF α -like growth factor and mature TGF α ,

peptide mapping was performed using the method of Cleveland. Immunoprecipitation of metabolically labelled conditioned media from MDA-MB-231, H8, and Rat-FeSrv cells was carried out with the R399 anti-TCF α polyclonal antibody.

- 5 Precipitates were analyzed on SDS-PAGE and the specific bands were electroeluted (30 Kd from MDA-MB-231 cells, 6 kDa from H8 cells, and 18 kDa from the Rat-FeSrv cells). These proteins were subjected to enzymatic treatment with N-glyconase and elastase. The precipitated bands sizes are
- 10 summarized in Table 2. The products were then subjected to a peptide digestion using 25 g/ml V8-protease. After complete digestion, the samples were analyzed by C18 reversed phase chromatography. Three major peptide peaks
- 15 eluted at different acetonitrile concentrations by reversed phase chromatography. However, the concentrations at which those peptides isolated from MDA-MB-231 cells eluted (16%, 18.7%, and 21.7%) were different from the peptides isolated from H8 and FeSrv cells (24%, 29%, and 32.6%). The peptide
- 20 elution pattern of the TGF α (6 Kd) derived from H8 cells and Rat-FeSrv cells was essential identical. The same results were obtained with 40 g of V8 protease, indicating that concentration of the enzyme was not responsible for the differential peptide cleavage. Moreover, in vitro
- 25 translation of mRNA derived from MDA-MB-231 cells and H8 cells was done, the resulting polypeptide had the same peptide mapping profile than the purified 30 kDa factor after treatment with N-glyconase and elastase. These results provide evidence that a precursor different than the "normal" TGF α precursor is translated from the mRNA of
- 30 MDA-MB-231 cells. Moreover, the above results indicate that the MDA-MB-231 derived TGF α -like polypeptide shares very few, if any, common peptide sequences with mature TGF α .

In order to characterize the cellular effects of the present 30 Kd glycoprotein, various experiments were conducted. The following Examples are provided solely for the purpose of illustrating the present invention and are not intended to be limitative.

Example 1

In order to characterize the cellular effects of the present 30 Kd glycoprotein ligand, its induction of tyrosine phosphorylation was assessed in the human breast cancer lines MDA-468 and SK-Br-3. Notably, MDA-468 cells have amplification and over expression of the EGFR gene and do not express erbB-2 receptor-like protein. SK-Br-3 cells have amplification and over expression of the erbB-2 gene as well as relatively elevated levels of EGFR. The 30 Kd ligand, TGF α and EGF were found to induce tyrosine phosphorylation in both cell lines an EGFR blocking antibody abolished the phosphorylation induced by the three growth factors in MDA-468 cells. This antibody did not, however, completely block the phosphorylation induced by the present 30 Kd ligand and SK-Br-3 cells. However, it did block the phosphorylation induced by TGF α .

From the above result, it appears that tyrosine phosphorylation of a protein is different from EGFR occurs in SK-Br-3 cells treated with 30 Kd factor. No phosphorylation was observed in untreated SK-Br-3 cells, and cells treated with the anti-EGFR antibody alone.

Example 2

In human mammary carcinoma cell line MDA-453, which over expresses erbB-2, but which has undetectable levels of the EGF receptor protein or mRNA, the 30 Kd ligand was observed to induce a significant increase in tyrosine phosphorylation in a dose dependent manner at concentrations ranging from 1.25 mg/ml to 50 mg/ml. By contrast, EGF and TGFU were unable to induce tyrosine phosphorylation in the 185 kDa range, at a concentration of 25 mg/ml. No phosphorylation was observed in untreated cells. Hence, from the above, a direct interaction between the 30 Kd ligand and the 185 Kd glycoprotein appears to occur.

Example 3

In order to determine the effects of the present 30 Kd ligand on the proliferation in colony formation of breast carcinoma cell lines, the following experiment was conducted.

Cells were treated with the present 30 Kd growth factor, EGF, TGF α and anti-erbB-2 antibody in order to inhibit the proliferation of SK-Br-3 cells.

It was observed that the anti-erbB-2 antibody inhibited the proliferation of the SK-Br-3 and MDA-453 cells by 60-70% but did not inhibit the proliferation of MDA-468 cells. Surprisingly, by exposing SK-Br-3, MDA-453 and MDA-468 cells to the 30 Kd ligand protein of the present invention, a 60-70% inhibition of cell growth was observed for all cell lines.

Inhibition of growth by the 30 Kd ligand protein was

reversed by an EGFR blocking antibody in MDA-468 cells, but not in SK-Br-3 or MDA-453 cells. This is an indication that the effects of the 30 Kd protein on SK-Br-3 and MDA-453 cells are not mediated through EGFR.

5 By contrast, the present 30 Kd glycoprotein exhibited no effect on MCF-7 cells, which have normal levels of EGFR and erbB-2. Additionally, EGF and TGF α inhibited the anchorage dependant growth of MDA-468 cells and SK-Br-3
10 cells, but not that of MDA-453 or MCF-7 cells. EGF-induced anchorage dependant growth inhibition of SK-Br-3 and MDA-468 cells was reversed by an anti-EGFR blocking antibody. In the presence of the 30 Kd glycoprotein, the growth inhibition of SK-Br-3, MDA-453 and MDA-468 cells was nearly complete.

15 The growth inhibitory property of the present 30 Kd ligand appears to be similar to that described for EGF on size whichever express EGFR such as A431 cells and MDA-468 cells.

Example 4

20 Further, the growth of CHO/erbB-2 transfected cells was inhibited by 70-80% after treatment with the present 30 Kd glycoprotein. No effect was observed on the CHO/DHFR control transfectants and the parenteral CHO line. TGF α at the same molar concentration did not exhibit any effect on
25 the proliferation of any of the three lines. Tyrosine phosphorylation and cell proliferation of the CHO/DHFR cells and the parenteral CHO cell line is not effected after treatments by the present 30 Kd ligand or TGF α .

Example 5: Cell Growth Inhibition by gp30

Sk-Br-3, MDA 453, MDA 468 and MCF-7 cells were plated in 24 well plates in IMEM (Biofluids) supplemented with 5% FCS. Parental CHO cells, and CHO cells transfected with the DHFR gene or the erbB-2 gene were plated in 24 well plates (Costar) in α -MEM (Biofluids) supplemented by 10% dialyzed FCS, 0.75 mg/ml G418 and Methotrexate (MTX) 50 nM for the CHO parental and CHO-DHFR CELLS for 250 nM for the CHO-erbB-2. After 24 hours media was removed and replaced with control serum free media (SFM) containing fibronectin, transferrin, hepes, glutamine, trace elements, and BSA, or SFM with the addition of 2.0 ng/ml gp30, 10 ng/ml recombinant TGF α (Genetech), or with 2.5 Mg/ml 4D5 specific anti-p185^{erbB-2} monoclonal antibody. Cells were grown in 90% confluence of control and counted. Each Group was assayed in triplicate. Results are shown as growth relative to control. The experiments were performed three times and the results were reproducible. The results are shown below in Table 2.

	SK-Br-3	MDA-453	CHO/erbB-2	CHO/DHFR	MDA-468	MCF-7
gp30	31	24	20	99	18	100
4D5 antibody	32	34	22	98	104	92
TGF α	73	91	89	95	79	105
Control antibody	87	91	87	94	92	99

In order to further describe the various aspects of the present invention, reference will now be made to the figures of the present specification.

Figure 1: Isolation of gp30

Part A illustrates the use of low affinity heparin chromatography. In particular, affinity chromatography of conditioned media from MDA-231 cells was performed on a heparin-sepharose column. Fractions were analyzed for EGF receptor binding activity of A431 cell membranes. Aliquots from the input media and from the fractions containing activity were analyzed by a 15% SDS-PAGE, followed by silver staining. Lane 1 shows unconcentrated conditioned media. Lane 2 represents the active fraction.

Part B illustrates the use of reversed-phase chromatography. Notably, the EGF/TFGA active fractions obtained after heparin-sepharose chromatography were chromatographed twice on a μ Bondapak C₃ column in 0.05% TFA. Samples were eluted with a steep gradient of acetonitrile. Fractions that showed EGF receptor binding activity were then rechromatographed and eluted with a shallow acetonitrile gradient. EGF competing activity was constantly eluted at a 25-30% acetonitrile gradient. The resulting fraction was analyzed on a 15% SDS-PAGE followed by silver staining. Sizes are shown in kilodaltons.

Figure 2: Detection of Phosphorylated Proteins in SK-Br-3 Cells

SK-Br-3 cells were grown in 90% confluence in 24-2311 plates (Costar). Cells were treated at 30°C with IMEM (lanes 1 and 2), IMEM containing 25 ng/ml recombinant TGF α (Genetech, CA) (lanes 3 and 4), and IMEM containing 5 ng/ml of gp30 (lanes 5 and 6), all of these in the presence (lanes 1, 4, 5) and the absence (lanes 2, 3, 6) of an anti-EGF receptor blocking antibody (Genetech, CA). After 20 minutes the media was removed and cells were lysed in 100 μ l of

sample buffer containing 1% SDS, 0.1% β -mercaptoethanol, 0.15 M Tris-HCl (Ph 6.8), 10% glycerol, 0.02 % bromophenol-blue, 1Mm EDTA, 2 Mm Pmsf and 42 Mm leupeptin. After 5 minutes at 95°C, 50 μ g of protein were loaded in a 7.5% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane for immunoblotting (Hoefer Scientific Instruments, California) by electrophoresis in a modified method of Towbin et al, using a electrophoretic transfer unit (Hoefer, TE 22). Electrophoretic transfer was carried out at room temperature for one hour at 125 Ma in a buffer containing 25 Mm glycine, 129 Mm Tris (Ph 8.3) and 20% methanol. Following transfer, the filter was blocked with 5% BSA in Tris-Buffered Saline containing 0.5% Tween 20. An antiphosphotyrosine antibody (Amersham) was reacted with the immobilized proteins in 5% BSA (Sigma RIA Grade). Immunocomplexes were detected by a goat anti-mouse antibody conjugated to alkaline phosphatase. Blots were then incubated with a color development substrate solution containing NBT and BCIP (Promega).

20 Figure 3: Detection of Phosphorylated Proteins in MDA-453 Cells

MDA-45e cells were grown to 90% confluence in 24-2311 plates (Costar) and treated at 37°C with IMEM (lane 1), IMEM containing 25 ng/ml of recombinant TGF α (Genetech, CA) (lane 10), or IMEM containing 1.25-40 ng/ml of gp30 (lanes 2-9). After 20 minutes media was removed and cells were lysed in 100 μ l of sample buffer as described in Figure 2. After 5 minutes at 95°C, 50 μ g of protein was loaded in a 7.5% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane for immunoblotting with an antiphosphotyrosine

antibody (Amersham) as described in Figure 2.

Figure 4: Phosphorylation of P185 Protein in Intact
CHO/DHFR, and CHO/erbB-2 Cells

Cells were grown to 90% confluence in 24-2311 plates
(Costar) in AMEM (Biofluids) supplemented with 10% dialyzed
FCS, 0.75 mg/ml G418, and Methotrexate (MTX) at
concentrations of 50nM (CHO parental and CHO-DHFR) OR 250 Nm
(CHO-erbB-2). CHO-DHFR (Fig. 4A) and CHO-erbB-2 (Fig. 4B)
cells, were treated at 37°C with control media supplemented
with 20 Mm Hepes (Ph 7.4) (A and B lanes 1 and 4), with 10
ng/ml of recombinant TGF α (Genetech, CA) (A and B lanes 2
and 5), and control media supplemented 2.0 ng/ml of gp30 (A
and B lanes 3 and 6). After 20 minutes, media was removed
and cells were lysed in 100 μ l of sample buffer (as
described in Figure 2). An anti-phosphotyrosine antibody (A
and B lanes 1 to 3) (Amersham) and an anti-erbB-2 antibody
(A and B lanes 4 to 6) (NEN), were reacted with the
immobilized proteins in 5% BSA (Sigma RIA Grade).
Immunocomplexes were detected as described for Figure 2.

Figure 5: P185erbB-2 Receptor Competition Assay in SK-Br-3
Cells

SK-Br-3 cells were plated in 24 well plates in IMEM
(Biofluids) supplemented with 5% FCS. After a wash with
binding buffer (DMEM/F12 pH 7.4, containing 1 mg/ml BSA, 10
Mm hepes and 20 Mm glutamine) cells were incubated for 30
minutes at 37°C with binding buffer. The EGFR were
saturated with 30 nM EGF for 2 hours at 4°C. p185 binding
studies were then performed for 3 hours at 4°C with 1 nM

iodinated 4D5 in the presence of various concentrations of unlabeled gp30 for 4D5. After the incubation, cells were washed 3 times with binding buffer and then solubilized with 1% SDS. No specific binding was determined with excess (100 nM) of unlabeled antibody. Each group was assayed in triplicate. The experiments were performed five times and the results were reproducible.

Figure 6: Inhibition of p185 Cross-linking with 4D5 Antibody of gp30

10 The binding assays were performed as described in Figure 5. Binding was performed with iodinated 4D5 (1 nM) alone (lane 1), in the presence of 100 nM unlabeled 4D5 (lane 2) and in the presence of 2nM gp30 (lane 3). 100 nM EGF were used as a control (lane 4). Cells were then
15 treated with a cross-linking agent EGS for 45 minutes at 4°C, then quenched by adding 0.1 ml of 20 Mm NH₄Cl. The solubilized cells were immunoprecipitated with a polyclonal antibody to the C-terminal domain of erbB-22 (Genetech, CA). The precipitates were analyzed on a 5% SDS-PAGE.

20 The 30 Kd glycoprotein of the present invention may thus be used advantageously to inhibit the growth of various types of adenocarcinoma cells which overexpress the erbB-2 oncogene and EGFR. Preferably, the present 30 Kd glycoprotein is used in inhibit the growth of adenocarcinoma
25 cells of breast, ovarian, gastric and lung tissue which overexpress the erbB-2 oncogene and EGFR.

In using the present 30 Kd glycoprotein to inhibit the growth of the above malignant cells in a mammal, preferably

a human, relatively low concentrations of the glycoprotein may be used. For example, an aqueous solution having a concentration of about 1-50 ng/ml may be conveniently administered to a patient such that a total of from about -
5 1-10,000 ng of glycoprotein are administered per day. It is preferred, however, if about 1-1,000 ng are administered per day.

The present invention thus relates to the use of the present 30 Kd TGF α -like glycoprotein in direct interactions
10 with EGFR and p185^{erbB-2}. Hence, in another aspect, the present invention provides conjugates of the 30 Kd glycoprotein ligand with either EGFR or p185^{erbB-2}. In still another aspect, the present invention provides diagnostic and therapeutic methods using these conjugates. Further,
15 the present invention provides a diagnostic test kit using the present conjugates.

In another aspect, the present invention relates to the preparation of monoclonal antibodies of gp30, and the use of these monoclonal antibodies to detect the presence of gp30
20 in patient sera.

As gp30 is known to be produced by MDA-MB-231 breast cancer cells, and is also likely to be produced by other adenocarcinoma cancer cells, the present invention also provides a method for detecting gp30 in patient sera.

25 Generally, in accordance with the present invention, the mere detection of either p185 or gp30 is a basis for concluding that the detected protein is being overexpressed. This conclusion, in turn, leads to a poor patient prognosis necessitating the use of more aggressive treatment of the
30 tumor.

In more detail, the present invention specifically first contemplates the use of conjugates of the 30 Kd glycoprotein and EGFR, and of the 30 Kd glycoprotein and p185^{erbB-2} in detecting the presence of adenocarcinoma cells
5 which overexpress either EGFR or erbB-2 oncogene. Preferably, the adenocarcinoma cells detected are of breast, ovarian, gastric and lung tissue.

Generally, the present conjugates may be used advantageously in a biochemical detection method in which
10 the 30 Kd glycoprotein ligand is bound to a surface and put into contact with aqueous solution containing a tumor portion containing cells which are suspected of overexpressing either EGFR or erbB-2 oncogene. This is conveniently done as either EGFR or p185^{erbB-2} may be found on the
15 cell surfaces. If such cells are present, either the EGFR or p185^{erbB-2} will become bound to the ligand. Thereafter, the aqueous solution is separated from the bound antiligand material, and the antiligand material may be conveniently detected with a known detection means associated therewith.
20 For example, an amplified enzymelinked immunoassay may be used. The surface to which the ligand is bound is treated with one or more agents for limiting the amount of non-specific binding. Such agents reduce the "noise" arising due to non-specific binding when interpreting the
25 assay.

In accordance with the above procedure, a diagnostic test kit may be constructed in a variety of ways.

For example, a test kit may be constructed to contain a vessel containing a test liquid having a surface to which
30 gp30 ligand is bound. This is preferably a multi-well test

plate. Also contained is at least one other vessel containing reagent solution. The agent for limiting non-specific binding may be incorporated within a solution of the kit or may have been used to treat the surface of the first vessel before it is supplied.

Then, a portion of the tumor or a tumor sample may be worked up into an aqueous solution and put into contact with the bound gp30.

In order to conveniently detect the overexpression of EGFR or erbB-2 oncogene in a human patient it is advantageous to use the well-known sandwich assay technique.

For example, one assay method and test kit which may be used in accordance with the present invention are described in U.S. Patent 4,668,639 which is incorporated herein in the entirety.

Hence, the present invention contemplates and is specifically directed to any diagnostic or therapeutic method for the detection of adenocarcinoma cells which overexpress EGFR or erbB-2 oncogene, which method uses the formation of a conjugate between the 30 Kd glycoprotein of the present invention and either EGFR or p185^{erbB-2}.

As noted above, the present invention also provides an assay and a test kit for the detection of gp30 using monoclonal antibodies to gp30.

It is noted that although either polyclonal or monoclonal antibodies can be used for this purpose, it is

preferred that monoclonal antibodies be used.

In such an assay, the monoclonal antibodies to gp30 are preferably bound to the microtiter or multi-well plate and exposed to patient sera suspected of containing gp30.

5 Upon detecting the presence of gp30 by a conventional detecting means, a conclusion of poor prognosis would be made necessitating the use of more aggressive treatment for the tumor. Importantly, however, the presence of the 30 Kd glycoprotein (gp30) in patient sera can be detected
10 utilizing either monoclonal or polyclonal antibodies in virtually any type of immunoassay. This includes both single-site or twosite or "sandwich" assays of the non-competitive types, as well as in traditional competitive binding assays.

15 With the above assay, a test kit is also provided. Generally, the kit contains a first container containing an antibody having specificity for gp 30 and a second container containing a second antibody having specificity for gp30 and being labelled with a reporter molecule capable giving a
20 detectable signal. The first antibody is immobilized on a solid surface.

The above assay and test kit for the detection of gp30 may be, respectively, conducted and constructed by analogy in accordance with U.S. Patent 4,921,790, which is
25 incorporated herein in the entirety.

The 30 Kd glycoprotein of the present invention is wellcharacterized by:

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- 1) being a heparin-binding protein;
- 2) being capable of binding to EGF receptor;
- 3) exhibiting cross-reactivity to antibodies to TGF α ;
- 4) being capable of cleavage by elastase; and
- 5) being capable of stimulating transforming activity in normal rat kidney (NRK) cells.

The polyclonal or monoclonal antibodies produced against gp30 may be produced in accordance with well-known techniques. For example, see Current Protocols in Molecular Biology, edited by F.M. Ausubel et al (Wiley 1987), in particular Chapter 11 on Immunology. Also, the immunoassays used in the assays and diagnostic test kits of the present invention are well known to the artisan as evidenced by the above treatise, and by the methods disclosed in U.S. Patent 4,921,790 which patent has been specifically incorporated herein in the entirety.

As described above, the diagnostic aspects of the present invention relate to the use of methods and test kits for the detection of either p185, EGFR or gp30. The detection of any one of these proteins may form the basis for a poor prognosis necessitating the use of aggressive treatment of one or more adenocarcinomas.

The present invention also relates to gp30, itself, and conjugates of gp30-EGFR and/or gp30-p185^{erbB-2}.

The therapeutic aspects of the present invention relate to the use of gp30 to inhibit the growth of adenocarcinoma cells which overexpress EGFR and/or erbB-2 oncogene.

5 Generally, the amount of gp30 to be administered as a therapeutic agent will be determined on a case by case basis by the attending physician. As a guideline, the extent of the adenocarcinoma, body weight and age of the patient are considered while up to about 10,000 ng per day may be used, generally not more than 1,000 ng per day of gp30 is
10 administered. It is preferred, however, if from about 5-500 ng per day are used. Notably, however, the above amounts may vary on a case-by-case basis.

While the present 30 Kd glycoprotein may be administered by itself, as a therapeutic agent, it may be
15 administered in combination with one or more other therapeutic agents. For example, the 30 Kd glycoprotein may be administered with any chemotherapeutic substance, growth inhibitor or immunestimulating substance. The present invention specifically contemplates such combinations.

20 Having now described the invention, it will now be apparent to one of ordinary skill in the art that many changes and modifications can be made to the above embodiments without departing from the scope and spirit of the present invention.

Claims

1. A method of inhibiting the growth of adenocarcinoma cells in a human, which cells overexpress the oncogene erbB-2 or EGFR, which comprises administering to said human
5 an amount of a 30 Kd glycoprotein effective to inhibit the growth of said cells.

2. The method of Claim 1, wherein said adenocarcinoma cells are adenocarcinoma cells of breast, ovarian, gastric or lung tissue.

10 3. The method of Claim 1, which comprises administering about 1-10,000 ng of 30 Kd glycoprotein per day to said human.

4. A 30 Kd glycoprotein obtained from MDA-MB-231 human breast cancer cells.

15 5. A conjugate of a 30 Kd glycoprotein obtained from MDA-MB-231 human breast cancer cells and a 185 Kd glycoprotein expressed by human erbB-2 oncogene.

6. A conjugate of a 30 Kd glycoprotein obtained from MDA-MB-231 human breast cancer cells and human EGFR.

20 7. A method of detecting a 185 Kd glycoprotein expressed by human erbB-2 oncogene or EGFR in human sera, which comprises:

a) contacting a solution of a tumor portion from a patient suspected of containing said 185 Kd glycoprotein or

EGFR with a bound 30 Kd glycoprotein obtained from MDA-MB-231 human breast cancer cells, thereby forming a conjugate of said 185 Kd glycoprotein or EGFR and said bound 30 Kd glycoprotein, and

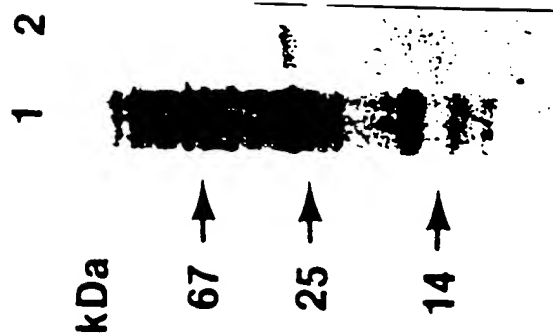
- 5 b) detecting or attempting to detect said formed conjugate by detecting means.

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FIG. 1B

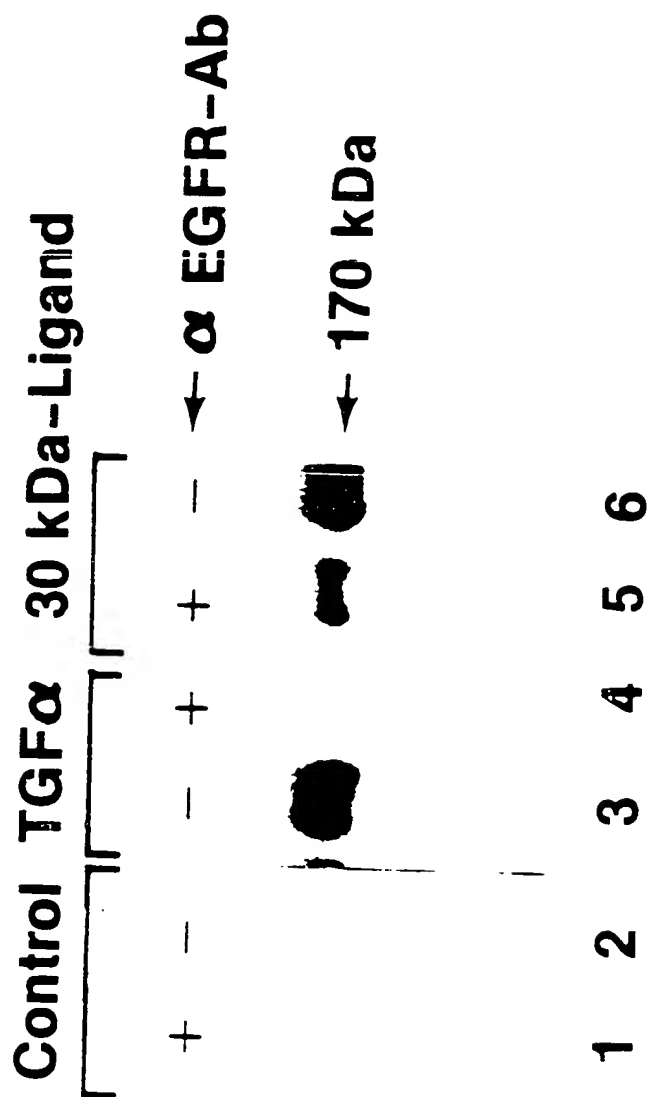


FIG. 1A



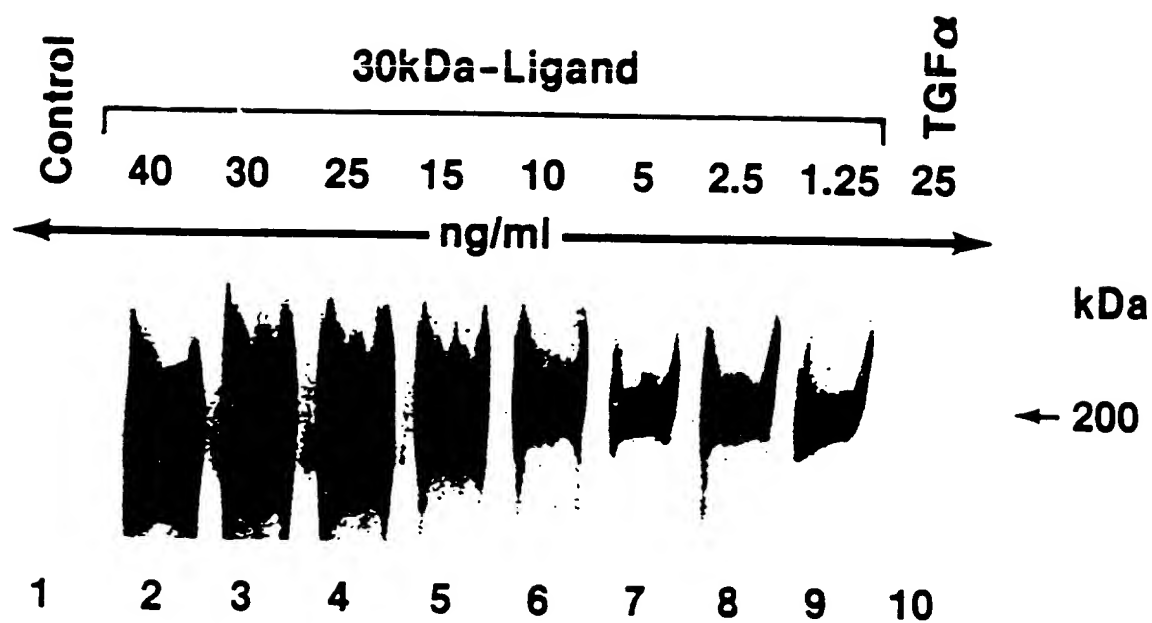
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FIG. 2



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FIG. 3



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FIG. 4A

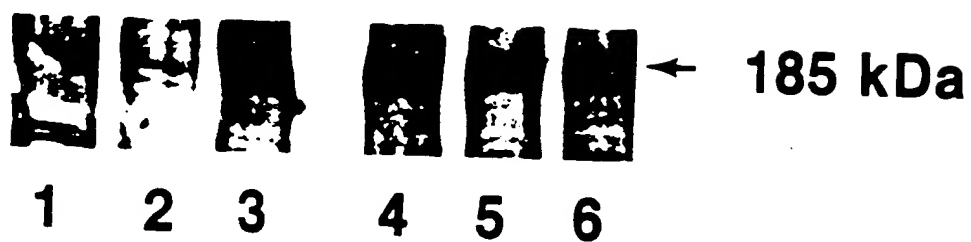
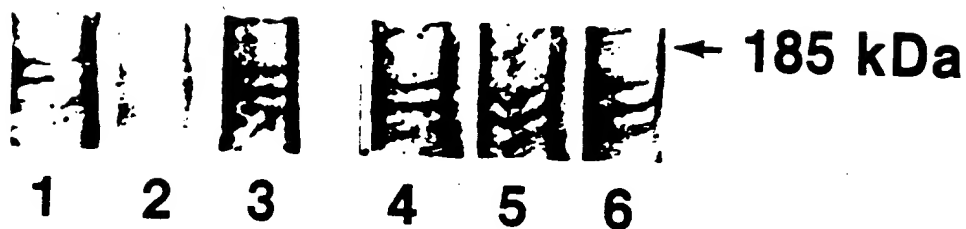


FIG. 4B



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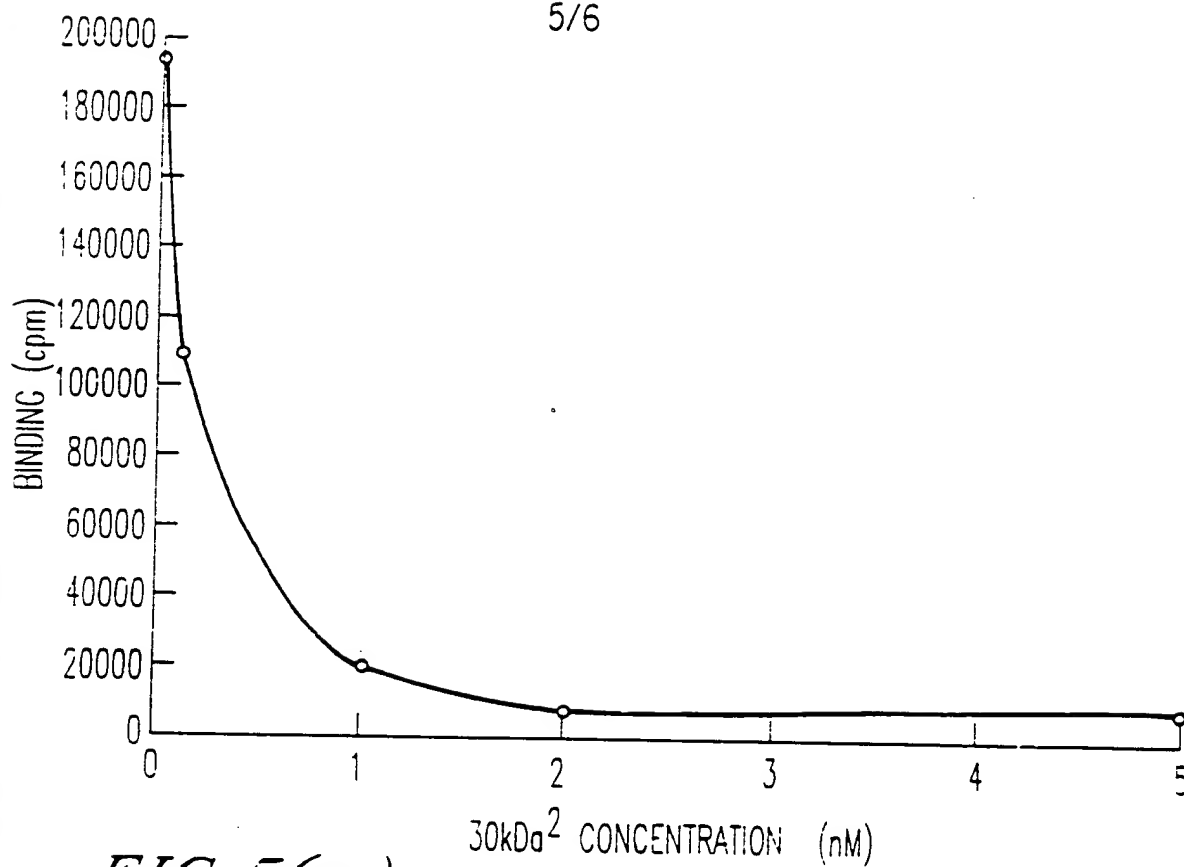


FIG. 5(a)

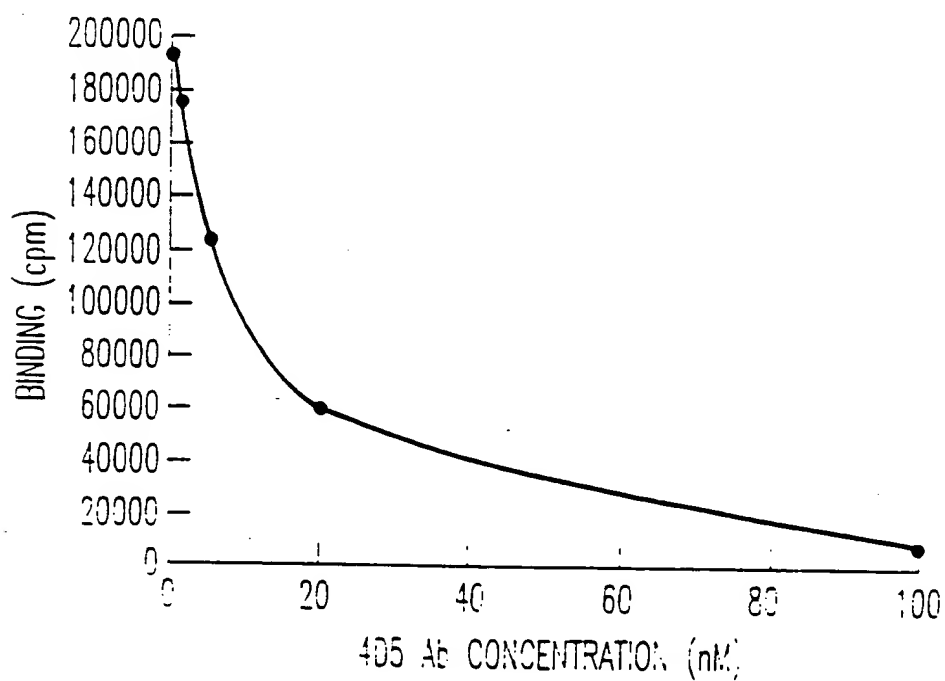
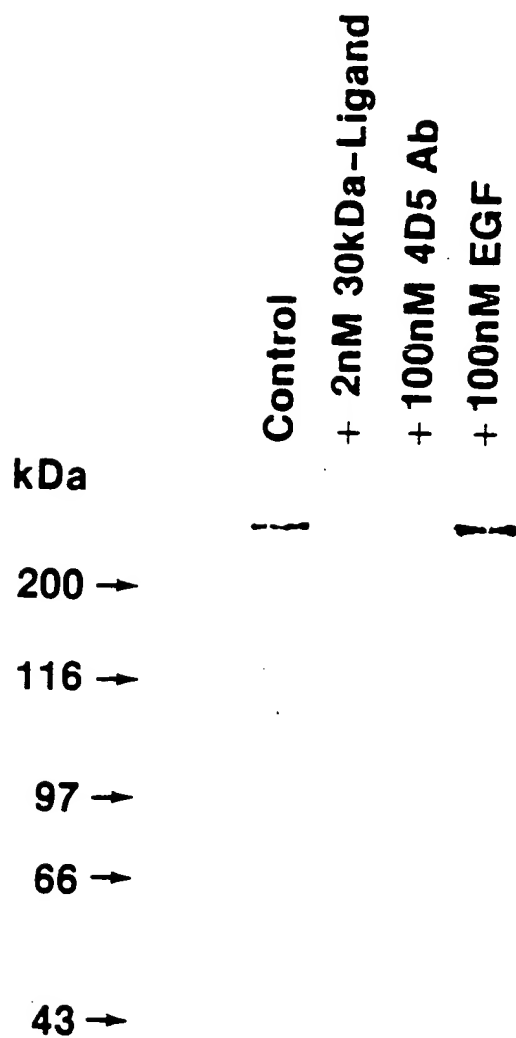


FIG. 5(b)

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**FIG. 6**

SUBSTITUTE SHEET